Supporting Information

Yin et al. 10.1073/pnas.0907010106

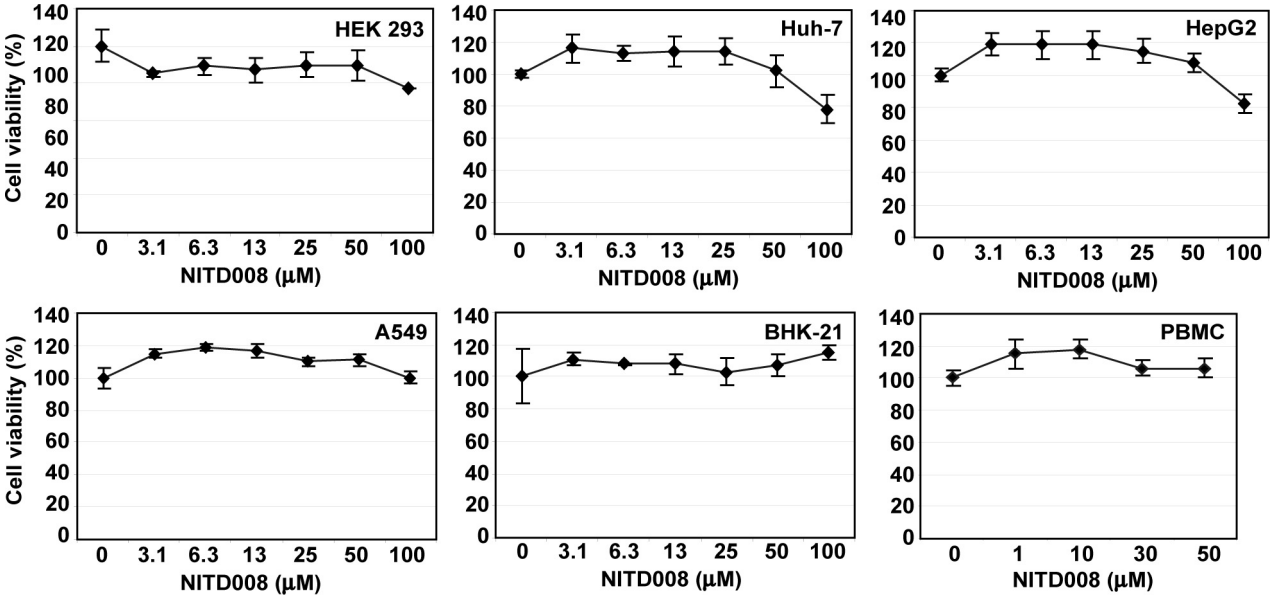

Fig. S1. Cytotoxicity analysis of NITD008. A panel of cell lines, including HEK 293 (human embryonic kidney cell), Huh-7 (human hepatoma cell), HepG2 (human liver carcinoma cell), A549 (human alveolar epithelial cell), BHK-21 (baby hamster kidney cell), and human primary PBMCs, was incubated with various concentrations of NITD008 for 48 h. A CellTiter-Glo luminescent cell viability assay was used to measure the intracellular level of ATP (to indicate cell viability) according to the manufacturer's protocol.

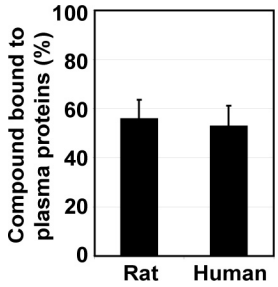

Fig. S2. Analysis of NITD008 binding to plasma proteins. NITD008 (0.5 mg/mL in methanol) was spiked into rat or human plasma at a final compound concentrations of 5 μg/mL (g/mL). The samples were vortexed for 10 sec, loaded onto an ultracentrifuge tube, and centrifuged at 436,000 $\times$ *g* for 140 min at 4 °C. The resultant plasma was separated into 3 distinct layers: a top layer containing lipoproteins and chylomicrons, a middle layer corresponding to macromolecule free plasma, and a bottom layer containing pelleted proteins. The middle layer fraction was collected and quantified for the amount of NITD008 using HPLC. The percentage of compound bound to plasma protein was estimated using the equation (1 − Final Compound Concentration After Centrifugation/ Initial Compound Concentration) $\times$ 100.

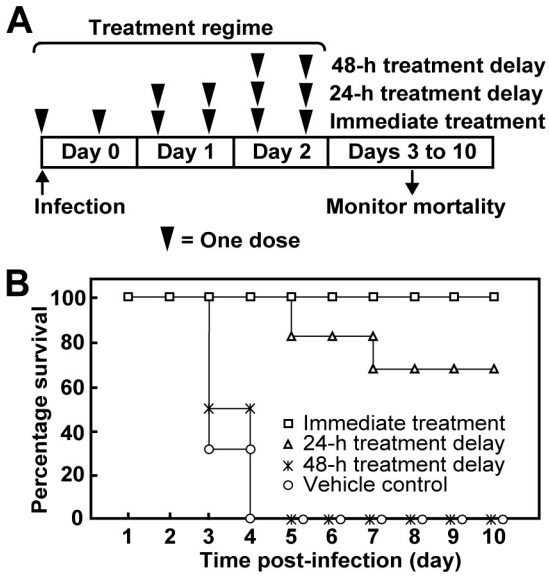

Fig. S3. Efficacy of delayed treatment in a dengue lethal mouse model. (*A*) Treatment regimen. AG129 mice were inoculated i.v. with DENV-2 strain D2S10 (see *Materials and Methods*). The infected mice were treated immediately or at 24 or 48 h postinfection (6 mice per group). Once the treatment began, the mice were dosed twice daily at 25 mg/kg per dosing. The treatment was stopped by the end of day 2. (*B*) Survival curve (see details in the legend for Fig. 3).

Table S1. Antiviral activity of NITD008 against 4 serotypes of DENV in various cell types

Cell type	Virus*	EC_{50} (μM)	Assay type
BHK-21	Dengue 1 (Hawaii)	0.16	CFI[†]
	Dengue 1 (MY10245)	0.82	CFI
	Dengue 2 (New Guinea C)	0.59	CFI
	Dengue 2 (New Guinea C)	0.87	Plaque assay[‡]
	Dengue 2 (New Guinea C)	0.68	CPE[§]
	Dengue 3 (MY21531)	0.65	CFI
	Dengue 3 (MY22366)	0.54	CFI
	Dengue 4 (MY22713)	0.16	CFI
A549	Dengue 1 (MY10245)	2.61	CFI
	Dengue 2 (New Guinea C)	1.64	CFI
	Dengue 2 (MY10340)	1.12	CFI
	Dengue 3 (MY21531)	0.65	CFI
	Dengue 3 (MY22366)	0.46	CFI
	Dengue 4 (MY22713)	0.70	CFI
Huh-7	Dengue 2 (New Guinea C)	1.64	CPE
PBMC (donor 1)[¶]	Dengue 1 (MY10245)	0.80	Plaque assay
PBMC (donor 2)	Dengue 1 (MY10245)	0.68	Plaque assay
PBMC (donor 3)	Dengue 1 (MY10245)	0.32	Plaque assay
PBMC (donor 4)	Dengue 1 (MY10245)	0.62	Plaque assay
PBMC (donor 5)	Dengue 1 (MY10245)	0.85	Plaque assay
PBMC (donor 6)	Dengue 1 (MY10245)	0.23	Plaque assay

*The virus strains are indicated in parentheses.

[†]Cell-based flavivirus immunodetection (CFI) is an ELISA-based assay that measures the amount of envelope protein in infected cells. Briefly, 2×10^4 A549 cells were seeded per well in a 96-well plate. The cells were infected with DENV [multiplicity of infection (MOI) = 0.3] on the following day and immediately treated with different concentrations of NITD008. The compound–virus mixture was incubated for 1 h with shaking every 10 to 15 min; the culture fluid was replenished with fresh medium containing compounds at various concentrations. On day 2 postinfection, the cells were washed with PBS, fixed with 100% methanol (vol/vol) at 4°C for 10 min, and detected for intracellular viral envelope protein by ELISA. The ELISA used mouse monoclonal antibody 4G2 (which specifically recognizes envelope protein of all 4 serotypes of DENV) and goat anti-mouse IgG conjugated with horseradish peroxidase as primary and secondary antibodies, respectively. EC_{50} values were calculated by nonlinear regression analysis.

[‡]BHK-21 cells were infected with DENV at an MOI of 0.3 in the presence of various concentrations of compound. Viral titers in culture medium on day 2 postinfection were determined by a plaque assay on BHK-21 cells. The plaques were visualized by staining the plaque assay plates with crystal violet on day 4 postinfection.

[§]CellTiter-Glo luminescent cell viability assay was used to measure the cytopathic effect of infected cells. Typically, 1×10^4 cells per well were seeded in a 96-well plate on day 1, compounds at various concentrations were added on day 2, and luciferase activity was measured to determine the cell viability on day 4.

[¶]Approximately 1×10^6 PBMCs in 100 μL of RPMI were infected with DENV at an MOI of 1 in the presence of compound for 1 h at 37°C. The DENV was preincubated with dengue IgG-positive serum (diluted by 10^4-fold in medium) on ice for 30 min to enhance the infection efficiency. After the infection, 300 μL of RPMI medium containing 5% FBS (vol/vol) and NITD008 was added to the culture. On day 2 postinfection, viral titers in culture fluids were determined by plaque assay on BHK-21 cells.

Table S2. Effect of human serum albumin (HSA) and α_1-acid glycoprotein (AAG) on EC_{50} values of NITD008

Assay condition*	EC_{50} (μM)
Without HSA or AAG	0.53
With 40 mg/mL HSA	1.34
With 2 mg/mL AAG	0.87
With 40 mg/mL HSA and 2 mg/mL AAG	1.41

*A549 cells were infected with DENV-2 (New Guinea C, MOI of 0.3) for 1 h without compound. One set of the infected cells was then incubated with medium containing various concentrations of NITD008; another set of cells was incubated with medium containing compound plus HSA and/or AAG. On day 2 postinfection, viral titers in culture fluids were determined by cell-based flavivirus immunodetection (CFI) assay, as described in the footnotes to Table S1.

Table S3. Effect of NITD008 on ligand binding to recombinant receptors*

In vitro recombinant protein binding assay	Species	IC_{50} (μM)
Adenosine 2A receptor	Human	>30
Adenosine 3 receptor	Human	>30
Adenosine transporter	Human	>30
Adrenergic β_1	Human	>10
Adrenergic β_2	Human	>10
Adrenergic β 3	Human	>30
α_1A receptor	Human	>10
α_2A receptor	Human	>10
α_2B receptor	Human	>10
α_2C receptor	Human	>10
Androgen receptor	Human	>30
Angiotensin II AT1 receptor	Human	>30
Benzodiazepine receptor	Rat	>10
Bradykinin 1 receptor	Human	>30
Bradykinin 2 receptor	Human	>30
Calcium channel (L) receptor	Rat	>10
Calcium channel (N) receptor	Rat	>10
Cannabinoid 1 receptor	Human	>10
Cholecystokinin A receptor	Human	>30
Cholecystokinin B receptor	Human	>30
COX-1 assay	Human	>30
COX-2 assay	Human	>30
CRF1 receptor	Human	>30
CRF2α -receptor	Human	>30
Dopamine D1 receptor	Human	>10
Dopamine D2 receptor	Human	>10
Dopamine D3 receptor	Human	>10
Dopamine D4.4 receptor	Human	>10
Dopamine transporter	Human	>10
Endothelin A receptor	Human	>30
Endothelin B receptor	Human	>30
Estrogen α -receptor	Human	>30
Estrogen β -receptor	Human	>30
GABA A receptor	Human	>10
GIP receptor	Rat	>30
Glucagon receptor	Human	>30
Glucocorticoid receptor	Human	>30
Ghrelin receptor	Human	>30
Histamine H1 receptor	Human	>10
Histamine H2 receptor	Human	>10
Histamine H3 receptor	Human	>10
Melanocortin MC3 receptor	Human	>30
Melanocortin MC4 receptor	Human	>30
Monoamine oxydase A	Human	>10
Motilin receptor	Human	>10
Muscarinic M1 receptor	Human	>10
Muscarinic M2 receptor	Human	>10
Muscarinic M3 receptor	Human	>10
Neurokinin NK1 receptor	Human	>10
NeuropeptideY Y1 receptor	Human	>10
NeuropeptideY Y2 receptor	Human	>10
Neurotensin NT1 receptor	Human	>10
Niacin receptor	Human	>10
Nicotinic (CNS) receptor	Human	>10
NMDA channel site receptor	Human	>10
Norepinephrine transporter	Human	>10
Opiate δ -receptor	Human	>10
Opiate κ -receptor	Human	>10
Opiate μ -receptor	Human	>10
Phosphodiesterase 3	Human	>10
Phosphodiesterase 4D	Human	>10
Serotonin 5-HT 1A receptor	Human	>10
Serotonin 5-HT 2A receptor	Human	>10
Serotonin 5-HT 2B receptor	Human	>10
Serotonin 5-HT 2C receptor	Human	>10
Serotonin 5-HT3 receptor	Human	>10
Serotonin transporter	Human	>10
Thromboxane A2 receptor	Human	>30
Vasopressin V1a receptor	Human	>10
Vasopressin V2 receptor	Human	>30

*All assays were performed using radioligand binding assays on mostly G-protein coupled receptors and on some transporters, ion channels, and enzymes.

Table S4. Effect of NITD008 on ligand binding to receptors expressed on cell surface*

Receptor assay	Species	Inhibition (%) at 10 μM
Adrenergic α_{1D}	Human	4
Adrenomedullin, AM_1	Human	4
Adrenomedullin, AM_2	Human	12
Anaphylatoxin C5a	Human	0
Androgen (testosterone), AR	Rat	−6
Angiotensin, AT_2	Human	−4
Angiotensin receptor-like I	Human	−3
Atrial natriuretic factor (ANF)	Guinea pig	1
Bombesin, BB1	Human	17
Bombesin, BB2	Human	4
Bombesin, BB3	Human	−2
Calcitonin	Human	11
Calcitonin gene-related peptide, $CGRP_1$	Human	−6
Calcium channel L-type, phenylalkylamine	Rat	−3
Cannabinoid, CB_2	Human	2
Chemokine, CCR1	Human	−1
Chemokine, CCR2B	Human	−1
Chemokine, CCR4	Human	−4
Chemokine, CCR5	Human	3
Chemokine, CXCR2 (IL-$8R_B$)	Human	−3
Cholinesterase acetyl, ACES	Human	5
COX-1	Human	14
Dopamine, D_5	Human	9
Erythropoietin, EPOR	Human	7
G protein-coupled receptor, GPR103	Human	−3
G protein-coupled receptor, GPR8	Human	−3
$GABA_A$ (chloride channel), TBOB	Rat	−9
Galanin, GAL1	Human	−2
Galanin, GAL2	Human	−4
Glutamate, AMPA	Rat	−1
Glutamate, kainate	Rat	5
Glutamate, NMDA, agonism	Rat	7
Glutamate, NMDA, glycine	Rat	−2
Glutamate, NMDA, polyamine	Rat	−12
Glycine, strychnine-sensitive	Rat	10
Histamine, H_4	Human	9
Imidazoline I_2, central	Rat	11
Inositol triphosphate, IP_3	Rat	−13
Leukotriene, BLT, LTB_4	Human	9
Leukotriene (cysteinyl), $CysLT_1$	Human	−7
Leukotriene (cysteinyl), $CysLT_2$	Human	−6
Melanocortin, MC_1	Human	−1
Melanocortin, MC_5	Human	−7
Melatonin, MT_1	Human	6
Melatonin, MT_2	Human	0
Monoamine oxidase, MAO-A	Human	5
Monoamine oxidase, MAO-B	Human	4
Muscarinic, M_3	Human	3
Muscarinic, M_4	Human	9
Neuromedin U, NMU_1	Human	3
Neuromedin U, NMU_2	Human	15
Nitric oxide synthase, endothelial (eNOS)	Bovine	17
Nitric oxide synthase, inducible (iNOS)	Mouse	2
Orexin, OX_1	Human	4
Orexin, OX_2	Human	7
Orphanin, ORL_1	Human	2
Phorbol ester	Mouse	17
Platelet activating factor (PAF)	Human	−4
Potassium channel, [K_A]	Rat	−13
Prostanoid CRTH2	Human	21
Prostanoid DP	Human	21
Prostanoid EP_2	Human	21
Prostanoid EP_4	Human	5
Purinergic P_{2X}	Rabbit	−6
Serotonin (5-hydroxytryptamine), $5\text{-}HT_4$	Guinea pig	−17
Serotonin (5-hydroxytryptamine), $5\text{-}HT_{5A}$	Human	3
Sigma, σ_1	Human	31
Sodium channel, site 2	Rat	17
Somatostatin, sst1	Human	5
Somatostatin, sst3	Human	7
Somatostatin, sst5	Human	4
Tachykinin, NK_2	Human	−15
Tachykinin, NK_3	Human	1
Transporter, choline	Rat	−1
Transporter, GABA	Rat	7
Transporter, monoamine	Rabbit	11
TNF, nonselective	Human	6
Urotensin II	Human	−4
VEGF	Human	13
Vasoactive intestinal peptide, VIP_1	Human	5
Vasopressin, V_{1B}	Human	5
Vitamin D_3	Human	−1

*The assays were performed using purified membrane fractions bearing the receptors of interest and radioligand. Results are presented as percentage of inhibition. Results with more than 50% inhibition or stimulation are deemed significant.